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## Introduction

Prohibitin, a potential tumor suppressor protein, was originally identified by its ability to induce G1/S arrest in human diploid fibroblasts. The prohibitin (Phb) gene was subsequently shown to be mutated in several sporadic breast tumors. We have shown that prohibitin binds Rb and represses all the five transcriptionally active E2Fs (Wang et al., 1999a). Prohibitin co-immunoprecipitates with both Rb and E2F1, and contacts each protein using different domains. Certain signaling cascades such as IgM stimulation of B cells reverses prohibitin-mediated repression of E2F1; Rb remains inert to this stimulus (Wang et al., 1999b). It had been shown earlier that microinjection of antisense oligonucleotides against prohibitin promotes entry into S phase (Nuell et al., 1991); (Jupe et al., 1995). Supporting this observation, we find that colony formation of various breast cancer cell lines is repressed by prohibitin. Repression by prohibitin requires the same domains that are used to bind to Rb and E2F; deletion of either of these domains abrogates prohibitin mediated growth arrest. Immunocytochemical studies indicate that prohibitin is highly expressed in neoplastic foci of various tumor types (Coates et al., 2001). It has been suggested that prohibitin associates with the IgM receptor in murine B lymphocytes (Terashima et al., 1994). In yeast, however, it has been found that prohibitin might associate with the mitochondrial inner membrane (Coates et al., 2001); (Berger & Yaffe, 1998); (Steglich, 1999); (Nijtmans et al., 2000). Most recently, prohibitin-2 was found to associate with and repress the nuclear estrogen receptor. Also known as REA for repressor of estrogen activity, this protein is unique in its ability to selectively bind the unliganded estrogen receptor and to maintain it in a repressed state (Delage-Mourroux et al., 2000); (Montano et al., 1999).

Given the growth suppressive function of prohibitin and its ability to repress E2F-mediated transcription, we set out to explore whether prohibitin also affects the apoptotic process. We show that the expression of prohibitin can protect cells from death induced by the chemotherapeutic drug camptothecin. While Rb family members are functionally inactivated during drug treatment, prohibitin levels are elevated. In addition, we find that prohibitin can functionally interact with the p53 tumor suppressor protein. We therefore speculate that prohibitin may have the dual ability to target both the Rb/E2F pathway and p53 pathway. We suggest that prohibitin provides another level of E2F regulation in situations where specific apoptotic stimuli upset the balance between survival and death.

### Body

Considerable progress has been made in the past year on elucidating the cellular functions of the prohibitin gene in breast cancer cell lines. These studies have led to two journal publications in 2002 (see below).

The first aim in the Statement of Work for this grant was to determine the level of apoptosis in breast cancer cell lines in the presence of chemotherapeutic drugs. These studies were proposed because initial experiments in B cells suggested that prohibitin may be protective against cytotoxic activity induced by particular drugs. A human B cell line (Ramos) stably over-expressing prohibitin was treated with camptothecin, Taxol, 5-fluorouracil, or tamoxifen, and the amount of apoptosis was determined. Surprisingly, cells over-expressing prohibitin exhibit about 50% less death upon treatment with camptothecin, a topoisomerase I inhibitor, compared to the parental cell line. Furthermore, studies using breast cancer cell lines also indicate a protective role of prohibitin during camptothecin treatment. BT 549 cells, which express high levels of endogenous prohibitin, exhibit 20% less death from camptothecin than ZR 751 cells, which have low levels. In addition, prohibitin protein levels increased after camptothecin treatment, while Rb was completely degraded. Correspondingly, the amount of E2F in complex with Rb as well as p107 and p130 decreased. Cyclin D levels remain constant, whereas cyclin E protein and message levels increase. Concomitant with dissociation with its repressors, E2F transcriptional activity increases in response to this drug, but this increase is attenuated in cells over-expressing prohibitin. The results of these studies were published in July, 2002 (Fusaro et al., 2002).

In addition to these findings, other experiments have shown that prohibitin is present in the nucleus of human fibroblasts, and a subset of it co-localizes with the Rb protein. Mechanistic studies have defined a role for the transcriptional co-repressors HDAC1 and N-CoR1 in mediating prohibitin's suppressive action on E2F function. The results of these studies were published in November, 2002 (Wang et al., 2002).

The second aim of the Statement of Work for this grant was to determine prohibitin levels and levels of other cell cycle regulatory molecules in breast cancer cell lines. In

the course of performing these studies, we have discovered a new and potentially exciting activity for prohibitin: a functional interaction with the p53 tumor suppressor protein. Prohibitin was found to associate with p53 *in vitro* by GST pull down assay. The specific domains involved in the binding are now being mapped. In addition, immunofluorescence experiments followed by confocal microscopy indicate that a significant proportion of cellular prohibitin is expressed in the nuclei of T47D and MCF7 breast cancer cells. Prohibitin and p53 co-localize in the nuclei of these cells. Furthermore, we find that the association of these proteins is altered *in vivo* after apoptotic stimulation. Prohibitin and p53 are redistributed out of the nucleus after cellular insult induced by 30  $\mu$ M campothecin for 4 hours. Co-transfection experiments indicate that prohibitin augments transcription from a p53-responsive reporter construct. This activation was observed in T47D cells and MCF7 cells, as well as Ramos B cells stably over expressing prohibitin. These results were confirmed by transfection of an antisense prohibitin construct, which ablated p53 activation. We are currently pursuing to delineate the mechanism by which prohibitin activates p53 function. Several potential mechanisms have already been tested and have not been shown to be involved. Prohibitin does not augment DNA binding by p53, nor cooperation with p300, nor affect MDM2 mediated repression of p53. We have no evidence that prohibitin effects p53 protein levels. Other potential mechanisms which we are in the process of testing include whether prohibitin affects the acetylation status of p53 or the acetylation of histones on p53 target promoters. These unpublished observations were presented in a poster at the DOD Era of Hope Breast Cancer Meeting in September, 2002, in Orlando, Florida.

The third aim in the Statement of Work was to determine the effect of prohibitin on gene expression. We initiated these studies by using the Ramos and Ramos-Phb cell lines described above to compare gene expression patterns. These cells were first serum starved for 72 hours, and then serum stimulated for 5 hours to promote cell cycle re-entry. Total cellular RNA was isolated and used to make cDNA, which was subsequently radiolabeled and hybridized to the Clontech Atlas 1.2 human cDNA expression array. This array was chosen because it contains about 1200 genes with known function in cell cycle control, apoptosis, or proliferation. cDNA from the Ramos cell line was compared to Ramos-Phb cells. Initial experiments indicate that the presence of prohibitin can alter gene expression pattern. Some genes which are expressed in the Ramos cell line are repressed in Ramos-Phb cells. These genes include E2F1, Platelet derived growth factor receptor, and Interferon response factor. On the other hand, some genes which are not expressed in Ramos cells are activated in Ramos-Phb

cells. These genes include BCL7b, cdk6, and cdk4I. We are currently in the process of extending these gene expression studies by utilizing Affimatrix human gene expression chips. An MCF7 cell line has been generated which contains a tetracycline inducible prohibitin gene. RNA will be collected from MCF7 cells before and after tetracycline treatment to study the effect of prohibitin on gene expression profiles in breast cancer cells. We expect that prohibitin will have an activating effect on some genes and a repressive effect on other genes. It will be interesting to note which of these genes are also regulated by E2F and/or p53, and which genes are regulated by prohibitin independantly of these transcription factors. These different classes of genes will likely provide novel insight into prohibitin function.

### Key Research Accomplishments

The following key research accomplishments in breast cancer cell lines have been supported by this award:

- Prohibitin is protective against apoptosis induced by the chemotherapeutic drug camptothecin.
- Prohibitin attenuates E2F activity during camptothecin induced apoptosis, when Rb family members are inactive.
- Prohibitin associates with p53 *in vitro* and *in vivo*.
- Prohibitin activates p53 transcriptional activity.

### Reportable Outcomes

The following reportable outcomes have been supported by this award:

- Manuscripts

1. **Fusaro**, G., Wang, S., and Chellappan, S. Differential regulation of Rb family proteins and prohibitin during camptothecin induced apoptosis. (2002). *Oncogene*, 21: 4539 - 4548.

2. Wang, S., **Fusaro**, G., Padmanabhan, J., and Chellappan, S. (2002). Prohibitin co-localizes with Rb in the nucleus and recruits N-CoR and HDAC1 for transcriptional repression. *Oncogene*, 21: 8388 - 8396.

- Presentations

"The Role of the Prohibitin Gene in Apoptosis of Breast Cancer Cells." Poster presented at the Department of Defense Era of Hope Breast Cancer Meeting, Orlando, Florida. September 25 - 28, 2002.



## Conclusions

Our data suggests several important functions for prohibitin in breast cancer cells. First, in the course of receiving genotoxic insult from camptothecin, cells degrade Rb while simultaneously increasing levels of prohibitin protein. In the presence of hyperproliferatory signals from transcription factors such as E2F, cells induce apoptosis to prevent uncontrolled growth. Prohibitin may protect cells from death by providing a means to rein in E2F activity and thus attenuate hyperproliferatory signals. Such an activity for prohibitin might have vital implications for the selection of drugs to treat breast cancer, because tumors that express high protein levels of prohibitin may be resistant to certain apoptosis inducing drugs.

Second, prohibitin interacts with the p53 tumor suppressor protein physically and functionally. These proteins associate *in vivo* and this association is altered in response to apoptotic signals from camptothecin. By activating p53 activity, prohibitin may aid in promoting p53 mediated cell cycle arrest or apoptosis in tumors which express both of these proteins.

We have evidence that prohibitin may intersect both the Rb/E2F pathway and the p53 pathway, providing a link between proliferation and growth control. Our studies are thus elucidating the mechanisms whereby prohibitin affects the chemotherapeutic response and may help in directing therapeutic strategies for patients with breast cancer.

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Wang, S., Fusaro, G., Padmanabhan, J., and Chellappan, S. (2002). *Oncogene*, **21**: 8388 - 8396.

## Differential regulation of Rb family proteins and prohibitin during camptothecin-induced apoptosis

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Prohibitin, a potential tumor suppressor, is known to induce growth suppression and repress E2F-mediated transcription. These growth regulatory functions of prohibitin require a physical interaction with the Rb protein. We now find that prohibitin protects cells from apoptosis mediated by camptothecin, a topoisomerase I inhibitor. Camptothecin treatment of Ramos B cells leads to the degradation of Rb protein and phosphorylation of its family members, p107 and p130. This correlates with an increase in the levels of cyclin E as well as the kinase activity associated with it. Inactivation of Rb leads to the dissociation and release of free E2F. We find also that E2F activity is induced upon camptothecin treatment, but this increase is absent in prohibitin overexpressing cells. It thus appears that prohibitin may be inhibiting apoptosis by downregulating E2F activity when Rb family members are inactive.

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**Keywords:** Rb; E2F; prohibitin; apoptosis; cell cycle

### Introduction

The E2F transcription factors play a major role in regulating the proliferation, differentiation and apoptosis of mammalian cells. There are six E2F family members, of which only five have transactivation domains; they along with their binding partners DP1 or DP2 induce genes necessary for S phase entry as well as DNA synthesis, promoting cell proliferation (reviewed in Dyson, 1998; Harbour and Dean, 2000). The transcriptional activity of the E2Fs is modulated mainly by the Rb family of proteins. Rb binds to and represses E2Fs 1, 2, and 3; p107 and p130 regulate E2F4 and E2F5. It is the functional hypo-phosphorylated form of Rb that binds and suppresses E2F activity, along with co-repressors like histone deacetylase 1 (HDAC1) and CtBP, as well as chromatin

remodeling proteins like Brg1 (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). A cascade of sequential phosphorylation events mediated by cyclin-cdk complexes leads to the inactivation of Rb, resulting in the release of free E2F, permitting S phase entry (Knudsen and Wang, 1996).

In addition to regulating cell cycle progression, E2F1 has also been shown to promote apoptosis in several experimental systems (reviewed in Phillips and Vanden, 2001). Overexpression of E2F1 promotes premature S phase entry and apoptosis in Rat-1 fibroblasts, with serum starved cells being particularly sensitive to death (Shan and Lee, 1994). Studies in knockout mice also support a role for E2F1 in apoptosis (Hunt *et al.*, 1997). The dissection of the pathways mediating E2F1 induced apoptosis has revealed that at least two different mechanisms exist: a p53 dependent pathway and a p53 independent pathway. The p53-dependent pathway is mediated by transcriptional induction of the human INK4A locus (Bates *et al.*, 1998). Accumulation of the p14ARF protein leads to increased levels of p53, because p14ARF binds to the MDM2/p53 complex and inhibits MDM2 mediated degradation of p53. Though it has been suggested that p53 independent apoptosis by E2F1 does not involve its transcriptional activation domain (Hsieh *et al.*, 1997), the p53 homologue p73 has been shown to be a transcriptional target of E2F1 and shown to be a mediator of p53 independent apoptosis (Irwin *et al.*, 2000; Lissy *et al.*, 2000). Thus, transcriptional activation of different target genes by E2F1 is a feature of both the p53 dependent and independent apoptotic pathways.

Beyond the classic Rb/E2F/DP1 interaction, other cellular proteins also bind to and regulate E2F function. Cyclin A-cdk2 kinase has been found to contact a region amino-terminal to the DNA binding domain leading to the phosphorylation of E2F1 and its eventual degradation (Xu *et al.*, 1994; Krek *et al.*, 1995; Kitagawa *et al.*, 1995). In addition, MDM2 has been reported to bind the transactivation domain and stimulate E2F activity while repressing p53 (Martin *et al.*, 1995). A recently identified mechanism of regulation of E2F1 is via acetylation on lysine residues. pCAF, a p300/CBP associated protein, acetylates E2F1 and augments DNA binding, protein half-life and transcriptional activity (Martinez-Balbas *et al.*, 2000).

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We have shown recently that a protein named prohibitin (Phb) binds and represses all the five transcriptionally active E2Fs (Wang *et al.*, 1999a). Prohibitin co-immunoprecipitates with both Rb and E2F1, and contacts each protein using different domains. Certain signaling cascades such as IgM stimulation of B cells reverses prohibitin-mediated repression of E2F1; Rb remains inert to this stimulus (Wang *et al.*, 1999b). It had been shown earlier that microinjection of antisense oligonucleotides against prohibitin promotes entry into S phase (Nuell *et al.*, 1991; Jupe *et al.*, 1995). Supporting this observation, we find that colony formation of various breast cancer cell lines is repressed by prohibitin. Immunocytochemical studies indicate that prohibitin is highly expressed in neoplastic foci of various tumor types (Coates *et al.*, 2001). It has been suggested that prohibitin associates with the IgM receptor in murine B lymphocytes (Terashima *et al.*, 1994). In yeast, however, it has been found that prohibitin might associate with the mitochondrial inner membrane (Coates *et al.*, 2001; Berger and Yaffe, 1998; Steglich *et al.*, 1999; Nijtmans *et al.*, 2000). Most recently, prohibitin-2 was found to associate with and repress the nuclear estrogen receptor. Also known as REA for repressor of estrogen activity, this protein is unique in its ability to selectively bind the unliganded estrogen receptor and to maintain it in a repressed state (Delage-Mourroux *et al.*, 2000; Montano *et al.*, 1999).

Given the growth suppressive function of prohibitin and its ability to repress E2F-mediated transcription, we set out to explore whether prohibitin also affects the apoptotic process. We show that the expression of prohibitin can protect cells from death induced by the chemotherapeutic drug camptothecin. While Rb family members are functionally inactivated during drug treatment, prohibitin levels are elevated. We therefore speculate that prohibitin provides another level of E2F regulation in situations where specific apoptotic stimuli upset the balance between survival and death.

## Results

### *Prohibitin protects cells from camptothecin induced apoptosis*

Since E2F1 has been demonstrated to be a pro-apoptotic transcription factor, we decided to examine whether prohibitin, a known repressor of E2F induced transcription, affected the response of cells to apoptotic stimuli. As an initial step, the B cell lymphoma line Ramos stably over-expressing prohibitin was generated and its response to four chemotherapeutic drugs was compared with that of the parental cells. The over-expression of prohibitin was checked by Western blotting, and the stably transfected cells contained about fivefold more prohibitin than the parental cells. Cells were treated with tamoxifen (an estrogen mimic), 5-fluoro-uracil (a thymidylate synthetase inhibitor),

paclitaxel (a microtubule depolymerization inhibitor), or camptothecin (a topoisomerase I inhibitor). A range of drug doses and treatment times were tested so that a significant number of cells underwent apoptosis. The level of apoptosis was assessed by annexin staining, followed by flow cytometry. As shown in Table 1, the overexpression of prohibitin did not significantly alter the number of annexin positive cells upon treatment with tamoxifen, 5-fluoro-uracil, or paclitaxel. However, upon treatment with 15  $\mu$ M camptothecin, the prohibitin over-expressing cells showed reduced levels of apoptosis compared to the parental Ramos line; 26% of the prohibitin over-expressing cells underwent apoptosis in response to camptothecin compared to 49% of the parental cells. These results were confirmed by repeating the annexin staining and using fluorescence microscopy to count the number of annexin positive cells *in situ*. These findings suggest that prohibitin can protect cells from camptothecin induced apoptosis.

To assess whether prohibitin affected receptor mediated cell death, the same two cell lines were treated with an anti-Fas antibody for 2 h. The two cell lines had similar levels of annexin positive cells when analysed by flow cytometry (Table 1) suggesting that prohibitin had no detectable effect on Fas mediated apoptosis. Thus, prohibitin provides specific protection to B cells from death induced by camptothecin, and does not serve as a general inhibitor of apoptosis.

Additional studies were done using human breast carcinoma cell lines that express different levels of endogenous prohibitin protein. We have shown previously by Western blotting that the cell line ZR751 has low levels of prohibitin protein, T47D an intermediate level, and BT549 a high level, relative to each other. These differing levels of prohibitin have an effect on E2F activity: those cells which contain more prohibitin have lower E2F activity than those with less prohibitin, as determined by transient transfection (Wang *et al.*, 1999a). When we measured the amount of apoptosis after treating these cells with 15  $\mu$ M camptothecin, it was found that the cells with the higher amount of prohibitin, T47D and BT549, were

**Table 1** Prohibitin represses camptothecin induced apoptosis

| Treatment       | Concentration | Time | % Annexin positive |               |
|-----------------|---------------|------|--------------------|---------------|
|                 |               |      | Ramos %            | Ramos (Phb) % |
| None            | —             | —    | 4.8                | 4.0           |
| Camptothecin    | 15 $\mu$ M    | 5 h  | 49.4               | 26.6          |
| Tamoxifen       | 60 $\mu$ M    | 24 h | 74.0               | 74.9          |
| Paclitaxel      | 300 nM        | 24 h | 42.8               | 41.6          |
| 5-Fluoro-uracil | 50 mM         | 16 h | 53.6               | 50.7          |
| Fas Antibody    | 50 ng/ml      | 6 h  | 49.7               | 47.1          |

Ramos B cells or Ramos cells stably over-expressing prohibitin were treated with camptothecin, tamoxifen, paclitaxel, 5-fluoro-uracil, or an anti-Fas antibody as indicated. Apoptosis was assessed by annexin staining followed by flow cytometry to determine the annexin positive cells. The values represent the average of two experiments

protected from death compared to the ZR751 line, expressing less prohibitin (Table 2). This result mirrors our findings with the Ramos cells, where cells over-expressing prohibitin showed a reduced amount of apoptosis.

The response of these breast cancer cell lines to receptor-mediated apoptosis was also assessed. The ZR751 and BT549 cells were treated with 50  $\mu\text{g/ml}$  of anti-TNF $\alpha$  for 6 h. The two cell lines exhibited similar levels of apoptosis as measured by counting the number of annexin positive cells *in situ* (Table 2). We conclude from these collective results that prohibitin does not affect receptor-mediated death by either the Fas or TNF pathways, but can confer protection to camptothecin in different cell lines. The potential mechanisms involved in this protection were next explored.

*Rb family members are inactivated during camptothecin induced death*

Because we have shown previously that prohibitin can both interact with Rb family members and repress E2F transcriptional activity, we next examined how drug

treatment affected the levels of Rb protein. For this purpose, whole cell extracts were prepared from Ramos cells treated with tamoxifen, camptothecin, 5-fluorouracil, or paclitaxel for 5 h and used for Western blotting using two different Rb antibodies. When an antibody specific for an internal region of Rb (amino acids 300 to 380) was used, a faster migrating, truncated species of Rb was evident (Figure 1a). When an antibody specific for the C-terminal region of Rb was used, no Rb could be detected after 5 h of camptothecin treatment (Figure 1a). Other studies have shown that a 5 kD portion of Rb at its C-terminus is cleaved during apoptosis induced by TNF $\alpha$  and other agents (Janicke *et al.*, 1996; Chen *et al.*, 1997). It appears that Rb might be undergoing caspase mediated degradation as a result of camptothecin treatment.

In order to understand the kinetics of camptothecin induced degradation of Rb, whole cell extracts were prepared from Ramos cells treated with the drug for 0, 1, 3, 5, or 8 h and examined by Western blotting. We found that Rb levels declined, to undetectable levels, after 5 h of treatment (Figure 1b).

Because we observed a degradation of Rb protein, we next examined whether the levels of the Rb family members p107 and p130 were affected in a similar fashion. Western blots of the same set of extracts showed that while Rb was degraded within 5 h of treatment, p107 and p130 levels were not affected significantly; but interestingly, they were shifted to a hyper-phosphorylated form (Figure 1b). We conclude from these results that camptothecin treatment inactivates the Rb family members, by either directly causing protein degradation, as in the case of Rb or by promoting phosphorylation of p107 and p130.

*Cyclin E levels rise in response to camptothecin*

Since we observed the hyper-phosphorylation of p107 and p130 in response to camptothecin, we next checked whether levels of cyclin D and cyclin E, which are

Table 2 Camptothecin-induced apoptosis in breast cancer cell lines

| Treatment    | Concentration       | Time | % Annexin positive |        |         |
|--------------|---------------------|------|--------------------|--------|---------|
|              |                     |      | ZR751 %            | T47D % | BT549 % |
| None         | —                   | —    | 2.5                | 1.0    | 1.8     |
| Camptothecin | 15 $\mu\text{M}$    | 7 h  | 21.3               | 1.6    | 2.8     |
| TNF $\alpha$ | 50 $\mu\text{g/ml}$ | 6 h  | 32                 | ND     | 31      |

Human breast carcinoma cell lines ZR751, which expresses low levels of endogenous prohibitin protein, T47D, which expresses an intermediate level, and BT549, which expresses high levels of endogenous prohibitin protein, were treated with camptothecin or TNF $\alpha$  as indicated. The number of dying cells was assessed by annexin staining followed by *in situ* fluorescence microscopy. ND = not determined

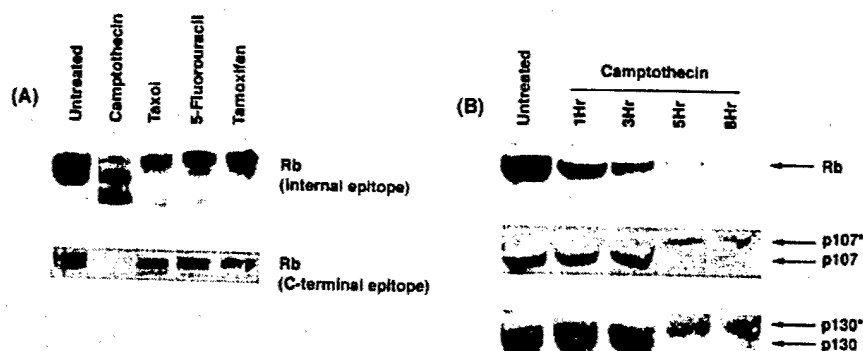


Figure 1 Rb family members are inactivated during camptothecin induced apoptosis. (a) Western blot analysis of Rb in extracts prepared from Ramos B cells treated with camptothecin, paclitaxel, 5-fluorouracil, or tamoxifen for 5 h. An anti-Rb antibody recognizing the internal amino acid residues was used. The same blot was stripped and re-probed with an antibody recognizing the C terminal domain of Rb. (b) Whole cell extracts were made from Ramos cells treated with 15  $\mu\text{M}$  camptothecin for 0, 1, 3, 5, or 8 h and a Western blot was done with an anti-Rb antibody recognizing the C terminal domain as in (a). The same blot was stripped and re-probed for p107 or p130. The asterisk indicates the hyper-phosphorylated form of the proteins

known to regulate these proteins by phosphorylation, were changed. The extracts used in the previous experiments were utilized again for Western blot analysis. We found that cyclin D1 levels remained constant in extracts treated with tamoxifen, camptothecin, 5-fluoro-uracil, or paclitaxel for 5 h compared to untreated cells (Figure 2a). However, when the same blot was stripped and probed for cyclin E, we found that its level increased significantly in the presence of camptothecin compared to the other drugs (Figure 2a).

To assess whether the changes in cyclin levels followed the same kinetics of phosphorylation observed in p107 and p130, Western blotting was performed on the Ramos extracts treated with camptothecin for 0, 1, 3, 5, or 8 h. While we did not observe a change in cyclin D1 protein levels, levels of cyclin E dramatically increased, following the same time kinetics as the changes seen in phosphorylation (Figure 2b). The changes seen in cyclin E corresponded with a slight increase in its kinase activity using histone H1 as a substrate in an *in vitro* kinase assay of camptothecin treated extracts (Figure 2c). Northern analysis of total cellular RNA prepared from camptothecin treated Ramos cells showed that the amount of cyclin E message increased by 5 h of drug treatment (Figure 2d) suggesting that the increase in cyclin E levels happens at the transcriptional level.

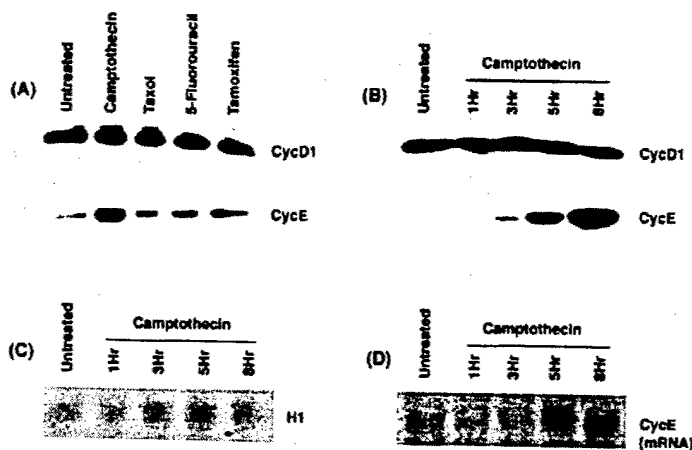
To ensure that the observed changes in cyclin levels were not due to differences in the cell cycle profiles of the asynchronous cell populations used, the camptothecin time course was repeated on cells that had been serum starved for 48 h. The Western blotting results were similar as observed for cycling cells (data not shown). Thus, camptothecin appears to induce cyclin E levels specifically.

### E2F loses association with Rb, p107 and p130

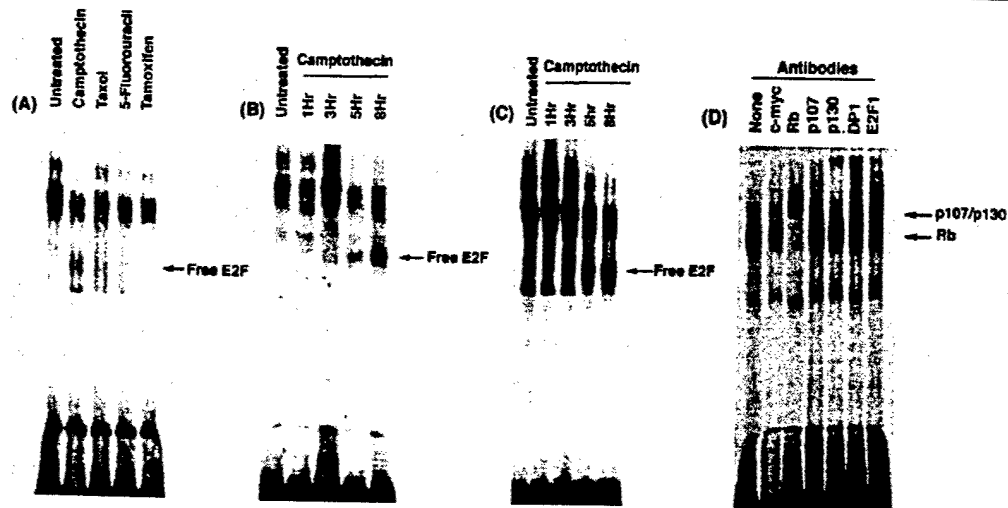
Given the functional inactivation we observed for Rb family members, we next explored the consequences of this inactivation on their binding to E2F. First, a gel-shift assay (EMSA) was performed using a radiolabeled probe derived from the adenovirus E2 promoter containing E2F binding sites. This probe was incubated with extracts prepared from cells treated with tamoxifen, camptothecin, 5-fluoro-uracil, or paclitaxel. When the bound complexes were resolved by non-denaturing gel electrophoresis, we observed an increase in the amount of unbound, free E2F in camptothecin treated cell extracts, but no changes in the extracts after treatment with the other drugs (Figure 3a). The extracts from cells treated with camptothecin for different time periods were also examined by gel-shift analysis. We found an increase in the free form of E2F appearing 5 h after treatment and persisting for at least 8 h (Figure 3b). This increase coincides with the functional inactivation of Rb family members.

To assess whether the Ramos cells over-expressing prohibitin also released free E2F, extracts from cells treated with camptothecin for 0, 1, 3, 5, or 8 h were used for gel shift experiments. We found that the prohibitin over-expressing cells exhibited an increase in the free form of E2F, similar to that seen in the parental cell lines (Figure 3c). Thus, inactivation of Rb family members results in release of free E2F in both cell lines. Antibody supershift assays were used to show the specificity of the identified complexes (Figure 3d).

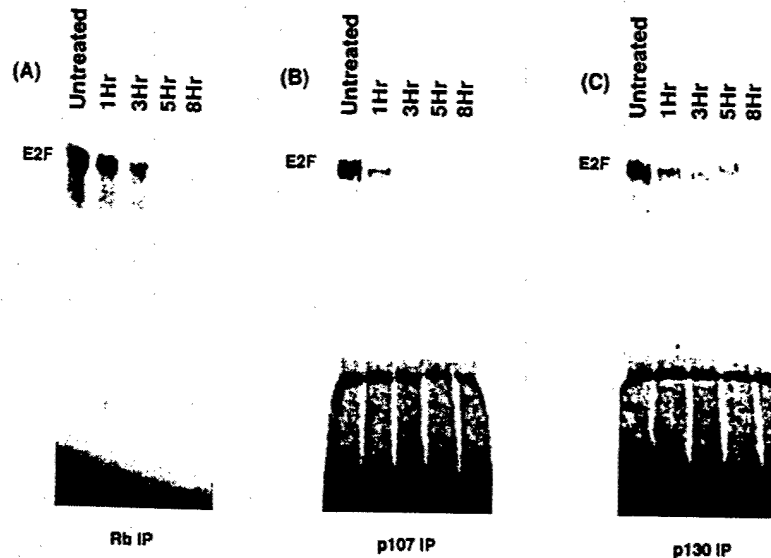
To evaluate the amount of E2F interacting with each Rb family member, co-immunoprecipitation followed by gel-shift was performed. The extracts from Ramos cells treated with camptothecin for different



**Figure 2** Elevated cyclin E protein level and activity in response to camptothecin treatment. (a) Whole cell extracts from drug treated Ramos extracts were prepared as in Figure 1 and a Western was performed for cyclin D1. The same membrane was stripped and re-probed for cyclin E. (b) Induction of cyclin E protein levels upon camptothecin treatment for 0, 1, 3, 5, or 8 h. The same blot was stripped and re-probed for cyclin D1. (c) Cyclin E kinase assay. Whole cell extracts from Ramos cells treated with camptothecin for the time points indicated were immunoprecipitated with a cyclin E antibody. Cyclin E associated kinase activity was assessed *in vitro* using histone H1 as a substrate. (d) Cyclin E Northern blot. Total cellular RNA was prepared from Ramos cells treated with camptothecin for the time points indicated and the level of cyclin E message was measured



**Figure 3** Free E2F is released upon camptothecin treatment. (a) Whole cell extracts from drug treated Ramos extracts were prepared as in Figure 1. A gel shift assay was performed using 10  $\mu$ g of extract incubated with a  $^{32}$ P-labeled probe containing E2F binding sites. The bound complexes were resolved on a polyacrylamide gel and visualized by autoradiography. Free E2F refers to E2F/DP complexes no longer associated with Rb family proteins. (b) Gel shift assay using 10  $\mu$ g of Ramos whole cell extracts prepared from cells treated with 15  $\mu$ M camptothecin for the time points indicated. (c) Gel shift assay using 10  $\mu$ g of extract from Ramos cells stably overexpressing prohibitin and treated with 15  $\mu$ M camptothecin for the time points indicated. (d). Antibody supershift assay. Extracts were pre-incubated with 4  $\mu$ l of the indicated antibodies for 4 h prior to addition of probe mixture. c-Myc antibody was used as a negative control

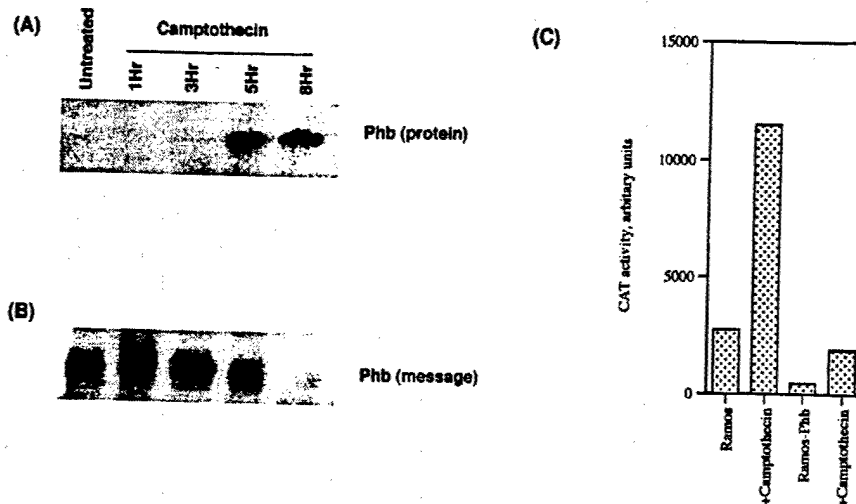


**Figure 4** E2F complexes are dissociated upon camptothecin treatment. One hundred and fifty  $\mu$ g of whole cell extracts prepared from Ramos cells treated with 15  $\mu$ M camptothecin for the time points indicated were immunoprecipitated with antibodies against (a) Rb, (b) p107, or (c) p130 and recovered on protein A beads. The immune complexes were released in sodium deoxycholate buffer, and presence of E2F detected by EMSA

time points were incubated with antibody for Rb, p107, or p130. The bound complexes were recovered on Protein A sepharose beads, washed, dissociated by incubation in sodium deoxycholate, and resolved on a polyacrylamide gel. In untreated cells, E2F was found to be present in complexes with all three Rb

family members. After 5 h of camptothecin treatment, however, the Rb/E2F interaction is completely lost (Figure 4a). E2F remains bound to p107 and p130 for a longer period of time compared to Rb, but the overall amount of bound complex is reduced over the time course of treatment (Figure 4b, c). From these





**Figure 5** Prohibitin inhibits E2F transactivation after camptothecin treatment. (a) Whole cell extracts from Ramos cells treated with 15  $\mu$ M camptothecin for the time points indicated were used in a Western blot for prohibitin. (b) Prohibitin Northern blot. Total cellular RNA was prepared from Ramos cells treated with 15  $\mu$ M camptothecin for 0, 1, 3 or 5 h and the level of prohibitin message was measured. (c) Ramos cells (columns 1 and 2) or Ramos cells stably transfected with prohibitin (columns 3 and 4) were transiently transfected with 10  $\mu$ g of an E2CAT reporter construct. Seventy-two hours after transfection, cells were treated with 15  $\mu$ M camptothecin for 5 h, lysed, and CAT activity was measured

results we infer that camptothecin leads to the disruption of the interaction between E2F and all three Rb family members.

#### *Prohibitin prevents E2F transactivation after camptothecin treatment*

Because of the inhibitory effect of prohibitin on camptothecin induced death, we next examined whether prohibitin protein levels were altered upon camptothecin treatment. Prohibitin levels in whole cell extracts made from Ramos cells treated with 15  $\mu$ M camptothecin for 0, 1, 3, 5 or 8 h were examined by Western blot analysis. In contrast to the degradation observed for Rb, prohibitin levels significantly increased after 5 h of drug treatment (Figure 5a). This increase is not occurring at the transcriptional level, because the level of prohibitin message remained unchanged during this treatment (Figure 5b). At 8 h of treatment, we observed a reduction in the amount of prohibitin message. This decrease is likely due to a degradation of the message. However, it remains possible that other mechanisms, such as a decrease in transcription, are causing the change.

In order to determine whether the generation of free E2F upon drug treatment resulted in increased transcriptional activity, a transient transfection was performed. Ramos cells were transfected with 10  $\mu$ g of a construct containing E2F binding sites fused to a CAT reporter. When these cells were treated with camptothecin for 5 h, a dramatic increase in CAT activity was seen compared to untreated cells (Figure 5c, columns 1 and 2). Interestingly, when this same experiment was repeated using Ramos cells stably over-expressing prohibitin, the amount of CAT activity was

reduced to almost basal level in the presence of camptothecin (Figure 5c, columns 3 and 4). We infer from these results that the increase in E2F transcriptional activity in response to this drug is ablated by the presence of prohibitin. Prohibitin may thus function to protect cells from camptothecin induced death by repressing E2F activity.

#### **Discussion**

Many cell cycle regulatory proteins have the dual function of regulating cell proliferation as well as apoptosis (reviewed in Blagosklonny, 1999). The E2F transcription factors, especially E2F1, are a prime example of this activity (reviewed in (Phillips and Vousden, 2001). Studies over the past few years have shown that E2F transcription factors play a significant role in promoting the G1/S transition (reviewed in Dyson, 1998; Harbour and Dean, 2000); interestingly, E2F1 over-expression also induces apoptosis under many circumstances (Shan and Lee, 1994). It became apparent that not only E2F1, but its main negative regulator, Rb, also responds to proliferative as well as apoptotic signals (Pan *et al.*, 1998; Morgenbesser *et al.*, 1994). Here we show that prohibitin, a novel regulator of E2F activity, also modulates apoptosis in response to specific signals.

Our current studies show that cells over-expressing prohibitin are resistant to camptothecin mediated apoptosis, but not apoptosis induced by other chemical agents. These observations are interesting in light of findings from other groups showing that over-expression of E2F1 in myeloid progenitor cells leads to sensitivity to the topoisomerase II inhibitor etoposide.

but not to other agents such as paclitaxel or 5-fluorouracil (Nip *et al.*, 1997). Indeed, increased prohibitin levels had no effect on receptor-mediated apoptosis either. Thus, prohibitin is capable of specifically targeting certain pathways leading to apoptosis; it may be imagined that prohibitin is blocking some earlier, specific events rather than late, shared events common to all apoptotic pathways like disruption of mitochondrial integrity and release of cytochrome *c*. It is not known whether prohibitin can affect these latter events in apoptosis, but it is an interesting possibility since prohibitin has been reported to be localized in the mitochondrial membranes as well (Coates *et al.*, 2001; Nijtmans *et al.*, 2000; Berger and Yaffe, 1998; Steglich *et al.*, 1999). Our experiments have shown prohibitin to be present ubiquitously in the cell, including the nucleus, where it co-localizes with Rb and affects transcriptional activity of E2F (unpublished data). Given the reported mitochondrial localization of prohibitin as well as the nuclear functions attributed to it, probably it is acting at multiple levels during the apoptotic process.

Our earlier studies have shown that prohibitin is capable of repressing E2F-mediated transcription very effectively, which might suggest that prohibitin is inhibiting apoptotic pathways by repressing the transcriptional activity of E2F1. The finding that prohibitin is affecting only specific and hence early signaling pathways leading to apoptosis can be explained by the transcriptional repressive properties of prohibitin. Investigations have revealed that E2F1 induces apoptosis by one of three generalized mechanisms: (1) inhibition of anti-apoptotic pathways, especially in receptor mediated cell death (Phillips *et al.*, 1999); (2) induction of pro-apoptotic genes like p73 and Apaf-1 (Irwin *et al.*, 2000; Lissy *et al.*, 2000; Muller *et al.*, 2001; Moroni *et al.*, 2001); and (3) stabilization of p53 levels by inducing p14ARF (Bates *et al.*, 1998). Gene array technology has identified novel target genes for E2F1 that have pro-apoptotic function, for example apaf1, caspase 3 and caspase 7 (Muller *et al.*, 2001) as well. Furthermore, disruption of the E2F1 gene causes increases in T cell levels, indicative of defects during the apoptotic program in T cell development (Field *et al.*, 1996; Yamasaki *et al.*, 1996). These studies strongly suggest that the transcriptional activity of E2F1 contributes to its apoptotic potential. It should be pointed out, though, that under certain circumstances, the activation domain of E2F1 is not necessary to induce apoptosis (Hsieh *et al.*, 1997). Overall, it appears safe to assume that E2F1 contributes to the apoptotic process by inducing multiple pro-apoptotic genes. It is possible that prohibitin may be inhibiting the apoptotic process by preventing the expression of these pro-apoptotic genes and experiments are underway to examine this possibility.

One of the intriguing observations is that all the three Rb family members are inactivated upon camptothecin treatment. It is likely that the cells are inactivating the Rb proteins to release free, transcriptionally active E2F to facilitate the apoptotic process.

The mechanisms behind the inactivation of the Rb family proteins reveal that while cyclin D levels remain stable, the level of cyclin E protein is enhanced. Interestingly, the cyclin E promoter has E2F binding sites and is regulated by the Rb-E2F pathway during cell cycle progression. Given the kinetics of Rb degradation and the induction of cyclin E, it appears that the increase in cyclin E levels could have resulted from increased E2F activity, and subsequently contributed to the phosphorylation of p107 and p130. This conclusion is drawn from the fact that Rb is already degraded before cyclin E levels are elevated.

Unlike Rb, we see no evidence that prohibitin is degraded during camptothecin induced death. Rather, prohibitin protein level increases. We have previously noted other differences between Rb and prohibitin in response to upstream signaling molecules (Wang *et al.*, 1999b). For example, co-transfection of cyclin D or cyclin E with Rb ablates the ability of Rb to repress E2F, but not prohibitin. The response of Rb versus prohibitin to viral antigens such as adenoviral E1A protein also differs. It might be of significance that the cells are elevating the levels of prohibitin, an E2F suppressor, that does not respond to cyclin E or protease degradation. Thus, the cells seem to elevate the levels of prohibitin which can suppress E2F activity when sufficient amounts of Rb family members are not present. This could be an attempt to maintain the cells in a quiescent state to facilitate the necessary repairs and adjustments needed to recover from the apoptotic stress and thus enhance the chance of survival. Though in our experiments prohibitin was unable to confer a complete protection against apoptosis, prohibitin may tilt the balance in favor of one response versus the other.

Although our initial interest in prohibitin stemmed from its similarity to Rb in repressing E2F activity and inducing growth arrest, our subsequent observations indicate that these two proteins respond to different signaling cascades. We infer from the current experiments that prohibitin not only acts as a repressor of E2F activity during normal cellular conditions, but may also aid in the decision between survival and death during times of apoptotic stimulation. Our continuing studies will try to elucidate which of these mechanisms are shared by the two proteins and which are divergent. These studies might lead to a more refined understanding of how different signals feed into E2F to control both cell cycle progression and apoptosis.

## Materials and methods

### Cell lines, transfections and vectors

The human Ramos B cell lymphoma line and Ramos cells stably transfected with full length prohibitin (Ramos Phb) were maintained in RPMI medium with 10% FBS. The T47D human breast carcinoma cell line was maintained in DMEM medium with 10% FBS and the ZR751 and BT549

human breast carcinoma cells were maintained in RPMI with 10% FBS.

Ramos cells stably overexpressing prohibitin were generated by transfection of pCDNA3-prohibitin (12 µg per 25 ml of cells) by electroporation. Cells were incubated overnight, and the next day the cell volume was doubled. Cells were incubated for 48 h. The culture was aliquoted into six-well culture plates. Cells were selected in G418 (40 µg/ml) for 3 days. Each cell culture was then diluted in half with further selection in G418 for another 3 days. This step was then repeated. The cells were then aliquoted into 96-well plates with an equal volume of G418 added to each well and cultured for 3 days. The cells were further serially diluted until clonal cell lines were established. The clones were confirmed by Western blot analysis. Stocks of cells were made from these lines, which were used in further experiments.

Transient transfection of Ramos cells and Ramos (Phb) cells was performed by electroporation using a Bio-Rad gene pulser at 250 V. Ten µg of E2CAT reporter plasmid was used in each transfection, along with 2 µg of a β-galactosidase expression vector as an internal control. Assays for β-galactosidase and chloramphenicol acetyltransferase activity were performed using standard protocols (Wang et al., 1999a).

#### Annexin staining, flow cytometry and immunofluorescence

Annexin staining was performed using the Annexin V-FITC Apoptosis Detection Kit 1 (Pharmingen) according to manufacturer's directions. Briefly, 500 000 cells were collected after drug or antibody treatment, washed two times with 1 × PBS, and resuspended in 500 µl of binding buffer. Cells were incubated with 4 µl of Annexin V-FITC at room temperature for 5 min, and then analysed by flow cytometry or immunofluorescence.

For visualization by immunofluorescence, cells were grown on 24 well plates and stained as described above. A Nikon Eclipse TE 300 microscope was used to count representative fields of cells scoring positive for FITC.

For flow cytometry analysis, stained cells were transferred to round-bottom tubes and analysed in a FACScalibur cell sorter using the FITC emission signal detector (FL1).

#### Whole cell extract preparation

Extracts were prepared by hypotonic shock. Ramos cells or Ramos (Phb) were collected by centrifugation at 1500 g for 10 min. Cell pellets were washed three times with cold PBS buffer. The packed cell volume was estimated, and cells were then resuspended in hypotonic buffer (10 mM KCl, 10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 0.5 mM DTT, 1 mM PMSF, 0.4 mM NaF and 0.4 mM Na<sub>2</sub>VO<sub>4</sub>) at 1.5 times the packed cell volume. Cells were allowed to swell for 15 min at 4°C. Cells were lysed by passing them 20 times through a syringe with a 22 G 1.5 needle. The lysate volume was measured, and a high salt buffer (1.6 M KCl, 20 mM HEPES, pH 7.0, 0.2 mM EDTA, 0.1 mM PMSF, 1 mM DTT, 0.4 mM NaF, 0.4 mM Na<sub>2</sub>PO<sub>4</sub> and 20% glycerol) was added to 1.66 times the lysate volume. The lysates were rotated at 4°C for 15 min and then ultracentrifuged at 100 000 g for 30 min at 4°C. The supernatant was collected and dialyzed in dialysis buffer containing 50 mM KCl, 20 mM HEPES pH 7.0, 0.1 mM EDTA, 0.1 mM PMSF, 1 mM DTT, 0.4 mM NaF, 0.4 mM Na<sub>2</sub>PO<sub>4</sub> and 10% glycerol for 30 min. The dialyzed cell extract was then spun at 15 000 r.p.m. for 10 min at 4 degrees. The supernatant was collected and stored at -80°C.

#### Drug and antibody treatments

Camptothecin, paclitaxel, 5-fluoro-uracil, and tamoxifen were purchased from Sigma. Anti-Fas mouse monoclonal antibody was purchased from MBL. TNFα recombinant protein was purchased from Promega. Ramos and Ramos(Phb) cells were treated with drugs as indicated in Table 1. BT549, T47D, and ZR751 cells were treated as indicated in Table 2.

#### Western blots and antibodies

Seventy-five µg of the whole cell extracts described above were boiled in SDS sample loading buffer and resolved on 8% polyacrylamide gels. The proteins were transferred onto nitrocellulose membranes by semidry transfer, blocked in 5% non-fat dry milk, incubated with the appropriate primary and secondary antibody, and detected by enhanced chemiluminescence assay (Amersham). The following primary antibodies were used: Rb mouse monoclonal (Oncogene Research Products Cat # OP28) specific for amino acids 300 to 380 of pRb; Rb rabbit polyclonal Ab (Santa Cruz Biotechnology, C15) mapping to the C-terminus of human pRb. The following antibodies were obtained from Santa Cruz: Cyclin E rabbit polyclonal (M-20); Cyclin D1 mouse monoclonal (HD11); p107 rabbit polyclonal (C-18). The Phb mouse monoclonal antibody was from NeoMarkers (Fremont, CA, USA). The p130 mouse monoclonal antibody was from Transduction Labs (R27020).

#### Immunoprecipitation

One hundred and fifty µg of whole cell extract was incubated with 5 µl of antibody to Rb, p107, or p130 for 1 h at 4°C. In a buffer containing 20 mM HEPES, pH 7.9, 40 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM NaF, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 0.5% NP-40, and 3 mg/ml BSA in a final volume of 100 µl. Then, 3 mg of protein A-sepharose beads were added in a 100 µl buffer volume and incubated for an additional 1 h at 4°C. The beads were washed five times with 700 µl of the same buffer. After washing, the beads were treated with 9 µl of 0.8% sodium deoxycholate made up in the same buffer to release the protein bound to the beads. The supernatant was treated with 1 µl of NP-40 and then used in subsequent gel shift analysis.

#### Gel-shift assay (EMSA)

An EcoRI/HindIII fragment of the adenovirus E2 promoter containing two E2F binding sites (TTTCGCGC) was end labeled with α<sup>32</sup>P-dATP by Klenow enzyme and used as a probe. The supernatants from the immunoprecipitation assays used above or 8 µg of whole cell extract was incubated with 0.2 ng of labeled E2F probe in a shift buffer made up of 20 mM HEPES, pH 7.9, 40 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM NaF, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 0.5% NP-40, 1 µg/ml of salmon sperm DNA and 10 µg/ml of BSA. After incubation at room temperature for 15 min, the reactions were separated on a 4% polyacrylamide gel in 0.25% TBE buffer at 300 V for 3 h at 4°C. The gel was then dried and the bands were detected by autoradiography. For supershift assays, the extract was first pre-incubated with 4 µl of antibody in 1 × shift buffer without NP-40, salmon sperm DNA, or BSA for 2 h on ice. The probe mixture was then added as above. The following antibodies were purchased from Santa Cruz: c-Myc mouse monoclonal (9E10), E2F1 rabbit polyclonal (C-20), and DP-1 rabbit polyclonal (K-20).

### In vitro kinase assay

Immunoprecipitation using the appropriate antibody was performed as described above. After the fifth wash, two additional washes were done in kinase assay buffer (50 mM Tris, pH 7.4, 10 mM MgCl<sub>2</sub>, and 1 mM DTT). The reactions were performed on the washed beads in a final volume of 30  $\mu$ l containing kinase assay buffer, 1.25  $\mu$ l of 2 mM ATP, 4  $\mu$ g histone H1 substrate (Sigma) and 10  $\mu$ Ci of  $\gamma$ -<sup>32</sup>P-ATP. Reactions were incubated for 15 min at 37°C, vortexed, and boiled. The boiled supernatant was separated on a 10% SDS-PAGE gel. Histone H1 phosphorylation was assessed by autoradiography.

### Northern blots

Total cellular RNA was prepared from asynchronously growing Ramos cells using the Total RNA Isolation System 1 (Promega) according to manufacturer's protocol. Twenty  $\mu$ g of total RNA was run on a 1% agarose formaldehyde gel and then stained.

To prepare the gel for transfer, it was first de-purinated in 0.25 M HCl for 10 min. It was then incubated in 50 mM NaOH for 25 min at room temperature, and then in 200 mM NaOAc, pH 4, for 2  $\times$  20 min at room temperature. The gel was blotted overnight in 10 $\times$ SSC on a MAGNA nylon membrane (Osmonics). The membrane was then rinsed in 2 $\times$ SSC and UV crosslinked (Stratalinker).

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## Prohibitin co-localizes with Rb in the nucleus and recruits N-CoR and HDAC1 for transcriptional repression

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The potential tumor suppressor protein prohibitin can prevent cell proliferation and this required its binding to the Rb protein. Prohibitin could repress the transcriptional activity of E2F family members and this required a part of the marked box region of E2F. The sub-cellular localization of prohibitin has been variously attributed to the mitochondria as well as the inner cell membrane. Here we show that a subset of prohibitin molecules are present in the nucleus where it co-localizes with the Rb protein. Deletion of a putative amino-terminal membrane-docking domain of prohibitin had no effect on its ability to suppress cell proliferation or inhibit E2F activity. Our experiments show that a 53 amino-acid stretch of E2F1 is sufficient for being targeted by prohibitin; fusion of this region to GAL4–VP16 construct could make it susceptible to prohibitin-mediated, but not Rb-mediated repression. Prohibitin, like Rb, could repress transcription from SV40 and major late promoters when recruited directly to DNA. Prohibitin mediated transcriptional repression required histone-deacetylase activity, but unlike Rb, additional co-repressors like N-CoR are also involved. Repression by prohibitin correlates with histone deacetylation on promoters and this was reversed by IgM stimulation of cells; IgM did not affect Rb-mediated repression or deacetylation of the promoters. Prohibitin thus appears to repress E2F-mediated transcription utilizing different molecular mediators and facilitate channeling of specific signaling pathways to the cell cycle machinery.

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**Keywords:** prohibitin; Rb; histone deacetylase 1; IgM; marked box

### Introduction

The E2F family of transcription factors play a major role in regulating mammalian cell cycle progression

and is capable of eliciting a wide array of biological functions including differentiation, transformation and apoptosis (Adams and Kaelin, 1996; Muller and Helin, 2000; Nevins, 1998). The transcriptional activity of E2F itself is regulated at multiple levels by a variety of mechanisms, to eliminate inappropriate activation causing unintended biological consequences (Dyson, 1998; Martinez-Balbas *et al.*, 2000). Many cellular genes required for the progression of the S phase have E2F sites in their promoter, and E2F activity is required for their expression (Adams and Kaelin, 1995). Activity of E2F is repressed by the interaction with the members of the Rb family of tumor suppressor proteins, and de-repression of E2F by inactivation of the Rb at the G1/S boundary facilitates S phase entry (Harbour and Dean, 2000b). It has been shown that over-expression or microinjection of E2F1 can induce S phase entry in quiescent cells, showing its potent proliferative capacity (Adams and Kaelin, 1996; Johnson *et al.*, 1993; Johnson and Schneider-Brousard, 1998).

The Rb protein represses E2F activity mainly by recruiting the histone deacetylase HDAC1 (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). HDAC1 has been shown to be involved in the Rb-mediated repression of many cellular promoters. It has been proposed that Rb might repress other promoters through other mechanisms, like recruitment of proteins such as CtBP, Ring1, DNMT1 and HP1 (Meloni *et al.*, 1999), preventing the formation of pre-initiation complexes (Ross *et al.*, 1999), or disruption of the interaction of E2F with co-activators. Rb protein interacts with HDAC1 directly, and unlike many other transcriptional repressors, additional co-repressors are not necessary (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). Chromatin remodeling proteins like Brg1/hBrm are also involved in the Rb-mediated regulation of the cell cycle: complexes of Rb with HDAC1 alone, or with HDAC1 and Brg1/hBrm regulate different stages of cell cycle (Zhang *et al.*, 2000).

We had observed that a potential tumor suppressor protein, prohibitin, could repress the activity of E2F family members (Wang *et al.*, 1999a,b). Prohibitin is growth suppressive, and its growth inhibitory function coincides with its ability to repress E2F activity. Rb- and prohibitin-mediated repression of E2F responds to different signaling pathways: molecules like adenovirus

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E1A and cyclin dependent kinases affect Rb and not prohibitin, while IgM stimulation of B cells releases prohibitin-mediated repression of E2F specifically (Wang *et al.*, 1999b). Similarly, Rb family members are inactivated upon camptothecin treatment while prohibitin levels are elevated and it remains functional (Fusaro *et al.*, 2002). Our earlier studies indicate that prohibitin targets the conserved marked-box region of E2Fs1-5 whereas Rb targets the transcriptional activation region of E2Fs1-3 (Wang *et al.*, 1999b). Here, we show that a subset of prohibitin molecules co-localize with Rb in the nucleus; further a part of the marked box region of E2F1 can sensitize other factors to prohibitin mediated repression. It appears that while prohibitin recruits HDAC1 to effect repression, co-repressors like N-CoR are involved. Finally, we show that prohibitin mediated repression correlates with histone deacetylation of two endogenous promoters and this changes upon IgM stimulation.

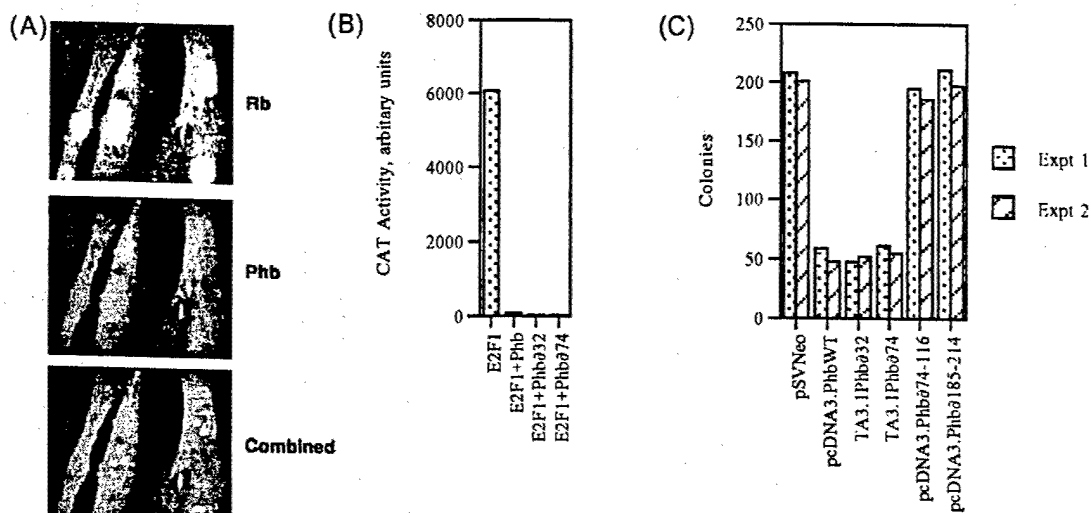
## Results

### *Prohibitin is present in the nucleus of cells and co-localizes with Rb*

Centrifugation experiments on rat liver lysates and immunohistochemistry on human tumor sections and rat ovaries had suggested that a subset of prohibitin maybe localized in the mitochondria or the cell membrane (Ikonen *et al.*, 1995; Thompson *et al.*, 1999). Similarly, prohibitin and related proteins have been reported to associate with the IgM receptor in rat B-cells (Terashima *et al.*, 1994). At the same time, it has been shown that a highly related protein,

prohibitin-2 can repress transcription mediated by steroid receptors (Delage-Mourroux *et al.*, 2000; Montano *et al.*, 1999). In addition, our experiments had shown that prohibitin binds to Rb and represses E2F-mediated transcription. Since these are nuclear functions, we decided to examine whether prohibitin is localized in the nucleus of human cell lines. A double immunofluorescence experiment was conducted on the human diploid fibroblast cell line HSF8 (Wang *et al.*, 1998); Rb was detected with a rabbit polyclonal antibody and a FITC conjugated secondary antibody. Prohibitin was detected using a mouse monoclonal antibody and a secondary antibody coupled to Rhodamine. As shown in Figure 1, most of the Rb is localized in the nucleus, though some of it could be detected in the cytoplasm as well. Discrete spots of intense Rb staining could be seen in the nucleus, as has been reported by other groups. Prohibitin appears to be ubiquitously distributed in the cell and a good amount of it is in the nucleus. Super-imposition of the two images show that prohibitin and Rb co-localize in the nucleus; some foci of intense Rb staining showed the presence of prohibitin as well.

It has been proposed that a putative hydrophobic membrane-docking region spanning residues 1-15 is responsible for the mitochondrial localization of prohibitin (McClung *et al.*, 1995). Since we find a portion of prohibitin in the nucleus, we examined whether this domain played a role in repressing E2F activity. A transient transfection experiment showed that deletion of the amino-terminal 32 or 74 amino acids did not affect the ability of prohibitin to repress E2F1-mediated transcription (Figure 1b). A colony formation assay was conducted to check whether



**Figure 1** (a) Prohibitin co-localizes with Rb in the nucleus. Localization of Rb (top) and prohibitin (middle) was examined using a double immunofluorescence technique. Superimposing the images show distinct foci in the nucleus where Rb and prohibitin co-localize. (b) A transient transfection experiment showing the ability of prohibitin lacking amino-terminal region to repress E2F1-mediated transcription. (c) A colony formation assay showing the growth suppressive properties of prohibitin. While deletion of the putative membrane-docking domain at the amino-terminus has no effect on growth suppression, deletion of the Rb-binding (74-116) or E2F1 binding (185-214) impairs growth suppression



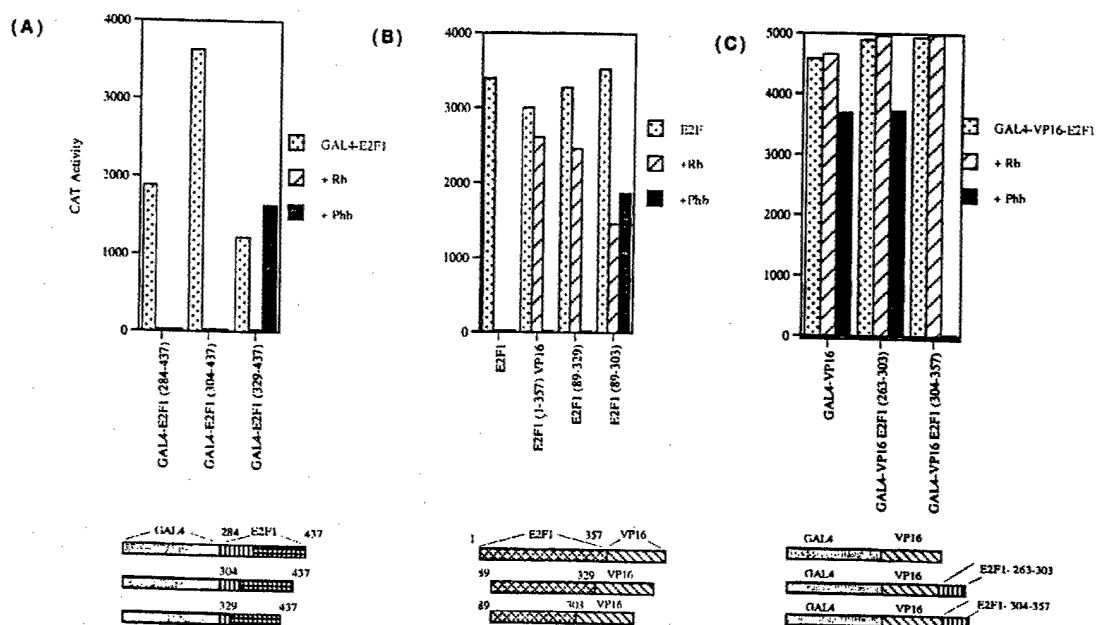
deletion of the amino-terminal residues affected the ability of prohibitin to inhibit cell proliferation. As shown in Figure 1c, transfection of a pSVNeo vector into T47D cells gave rise to approximately 200 neomycin resistant colonies after 2 weeks. Transfection of full length prohibitin reduced the number of colonies to around 55, indicating growth suppression. Interestingly, over-expression of prohibitin constructs lacking either 32 or 74 amino-terminal residues suppressed cell proliferation to a comparable extent; but as we had demonstrated earlier, the Rb and E2F binding regions of prohibitin (residues 74-116 and 185-214 respectively) are necessary for growth suppression. It thus appears that the amino-terminal domain of prohibitin is not needed for arresting cell proliferation. Since the nuclear functions of prohibitin appear to be involved in growth suppression, attempts were made to study the underlying molecular mechanisms further.

# Marked box of E2F1 confers sensitivity to prohibitin-mediated repression

Our earlier studies had shown that while a GAL4-VP16 fusion protein cannot be repressed by prohibitin, constructs carrying different regions of E2F1 could be repressed by prohibitin. Thus GAL4-E2F1 (283-437) and E2F1 (1-357)-VP16 fusion proteins could be repressed by prohibitin, suggesting that prohibitin targets the region 283-357 shared between the two

constructs (Wang et al., 1999b). Supporting this hypothesis, prohibitin could not inhibit the transcriptional activity of an E2F1 molecule that lacked the region 304-357 in transient transfection experiments. Experiments were designed to examine whether this region of E2F1 could render other transcription factors sensitive to prohibitin-mediated repression. As a first step, we generated chimeras of E2F1 with GAL4 DNA binding domain and tested their ability to respond to prohibitin and Rb. These chimeras had the varying lengths of the E2F1 marked box and the entire transcriptional activation domain of E2F1 fused to the GAL4 DNA-binding domain. As shown in Figure 2a, prohibitin could repress a fusion of GAL4 with E2F1 (283-437) and E2F1 (304-437), but could not affect a fusion carrying E2F1 (329-437). This suggested that prohibitin could repress GAL4-E2F1 mediated transcription only when residues 303-357 of E2F1 was present. In contrast, Rb was able to repress all the GAL4-E2F1 fusions, since they all had the transcriptional activation domain.

Complimentary studies were conducted on fusions of E2F1 with the VP16 activation domain; these constructs had the entire DNA-binding domain of E2F1 and varying lengths of the marked box region fused to VP16 transcriptional activation domain. Prohibitin could repress a fusion of E2F1(1-357) or E2F1(89-329) with VP16 activation domain (Figure 2b); but interestingly, prohibitin could not repress a construct carrying E2F1(89-303) fused to VP16AD.



**Figure 2** (a) Gal4 DNA binding domain was fused to the indicated regions of E2F1 and transfected with a pGE1B5GAL4 CAT reporter; repression mediated by Rb and Phb is shown. (b) E2F1 or different regions of E2F1 fused to a VP16 activation domain as shown were transfected along with an E2CAT reporter, and the effect of Rb and Phb measured by CAT assay. (c) pGE1B5GAL4-CAT was induced with GAL4-VP16 or GAL4-VP16 fused to regions 263-303 or 304-357 of E2F1, and the effect of Rb and Phb measured



Rb could not repress any of these constructs. This experiment suggests that prohibitin-mediated repression of these chimeras required the marked box region of E2F1 and was independent of the DNA binding and transcriptional activation domains.

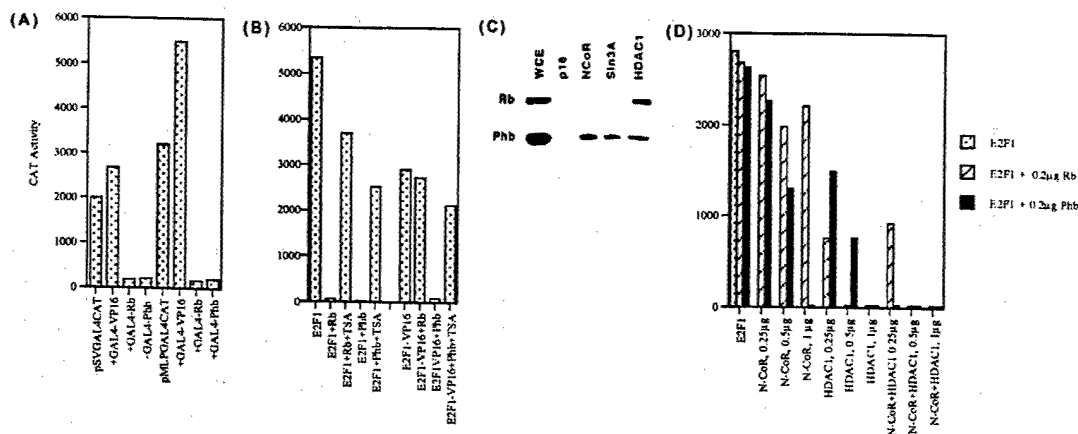
Examined next was whether the region 304–357 of E2F1 can render a transcription factor responsive to prohibitin mediated repression. As described earlier, transcription of a GAL4–CAT reporter induced by a GAL4–VP16 fusion protein cannot be repressed by prohibitin. We generated GAL4–VP16 fusion proteins that had residues 263–303 or 304–357 of E2F1 fused to their carboxy-terminal; these two constructs stimulated the transcription from a GAL4 reporter efficiently, comparable to GAL4–VP16 (Figure 2c). Co-transfection of prohibitin did not repress GAL4–VP16, or its fusion with residues 263–303 of E2F1. In contrast, prohibitin was able to repress transcription of GAL4–VP16 fused to the residues 304–357 of E2F1. As expected, Rb could repress none of the above constructs since it specifically targets the transcriptional activation domain of E2F1. It appears that the region 304–357 is sufficient for prohibitin-mediated repression of E2F1, and this region of E2F1 can confer prohibitin response to other transcription factors.

#### Prohibitin recruits HDAC1 to repress transcription

It has been shown that Rb protein could repress transcription effectively when recruited to a promoter either through E2F1 or through fusion with GAL4 DNA binding domain (Adnane *et al.*, 1995; Weintraub *et al.*, 1992). Since prohibitin is also a repressor of E2F activity, we examined whether prohibitin can also repress transcription when recruited to a promoter

independent of E2F1. A fusion of prohibitin with the DNA binding domain of GAL4 was generated for this purpose. Two CAT reporters, driven by SV40 early or adenovirus major late promoters each carrying GAL4 DNA binding sites were transfected into T47D cells. Co-transfection of a GAL4–VP16 construct could stimulate both the reporters above basal levels (Figure 3a). But co-transfection of a GAL4–Rb construct or a GAL4–prohibitin construct could lead to a marked repression of both the reporters. This was dependent on the two proteins being physically recruited to the promoter, since there was no repression when Rb and prohibitin were not fused to GAL4 (data not shown). This shows that prohibitin, like Rb, can repress transcription when recruited to a promoter, even independent of E2F1.

Since it had been shown that Rb recruits the histone deacetylase HDAC1 for transcriptional repression, we first examined whether it is involved in prohibitin mediated transcriptional repression as well (Luo *et al.*, 1998). First we examined whether the histone deacetylase inhibitor Trichostatin A can affect prohibitin-mediated transcriptional repression. T47D cells were transfected with an E2CAT reporter whose activity was induced by E2F1 or E2F1–VP16. Co-transfection of Rb or prohibitin could repress E2F1 mediated transcription (Figure 3b); interestingly, treatment of the transfected cells with 200 nM TSA could reverse both Rb and prohibitin mediated repression. To rule out the possibility that TSA is functioning through endogenous Rb–HDAC1 complexes rather than prohibitin, a similar experiment was conducted where E2F1–VP16 was used instead of E2F1. TSA could effectively reverse prohibitin-mediated repression of this construct as well; since Rb could not repress this construct, it may be concluded that TSA is affecting



**Figure 3** (a) pSVGAL4CAT or pMLPGAL4CAT were co-transfected with GAL4 fusions of VP16, Rb or prohibitin. While GAL4–VP16 could induce both the reporters, the fusions with Rb and Phb repressed transcription. (b) Effect of Trichostatin A on Rb and Phb-mediated repression. E2CAT reporter was co-transfected with E2F1 or E2F1–VP16 along with Rb and Phb as indicated. Treatment of transfected cells with TSA could relieve both Rb and Phb-mediated repression. (c) Daudi whole cell extracts were immunoprecipitated with the antibodies indicated on top, and the presence of Rb and prohibitin examined by Western blotting. (d) E2CAT reporter was co-transfected with E2F1 and 0.2  $\mu$ g of prohibitin or Rb. Effect of co-transfecting 0.25, 0.5 or 1  $\mu$ g of N-CoR, HDAC1 or a combination of both along with Rb or prohibitin is shown

prohibitin-mediated repression independent of Rb-HDAC1 complexes.

It has been reported that Rb interacts with HDAC1 directly independent of transcriptional co-repressors like N-CoR or Sin3A (Amann *et al.*, 2001; Brehm *et al.*, 1998; Luo *et al.*, 1998; Lutterbach *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998), and we made attempts to see whether it is true for prohibitin as well. Whole-cell extracts were immunoprecipitated with antibodies to c-myc, N-CoR, Sin3A or HDAC1 and the presence of Rb in the precipitates was assessed by Western blotting (Figure 3c). As reported earlier, there was no detectable amount of Rb associated with N-CoR, Sin3A or c-myc (which was the negative control). But a significant amount of Rb could be detected in association with HDAC1, confirming that Rb binds to HDAC1 directly. The blot was stripped and re-probed with an anti-prohibitin antibody, to check the presence of prohibitin. As can be seen from Figure 3c, there was a detectable amount of prohibitin associated with N-CoR, Sin3A as well as HDAC1, even when the immunoprecipitations were done under stringent conditions. This suggested that even though prohibitin recruits HDAC1, additional co-factors like N-CoR are involved.

The functional significance of the two interaction patterns was next examined by transient transfection experiments. The strategy was to test whether N-CoR and HDAC1 could synergize with Rb and prohibitin to repress transcription. T47D cells were transiently transfected with E2CAT and E2F1 to obtain a significant amount of transcription. Co-transfection of a minimal amount (0.2  $\mu$ g) of prohibitin or Rb did not cause a significant reduction in the transcription (Figure 3d). An increasing amount of N-CoR was co-transfected along with the low amounts of prohibitin: 0.25  $\mu$ g of N-CoR had no effect, but 0.5  $\mu$ g could reduce the levels of transcription by half. When 1  $\mu$ g of N-CoR was co-transfected, there was a complete repression of E2F activity, suggesting that N-CoR can synergize with prohibitin to repress transcription. Similar results were obtained with HDAC1 as well, with 0.25  $\mu$ g of HDAC1 having no effect on transcription and 1  $\mu$ g effectively synergizing with 0.2  $\mu$ g of prohibitin to bring about complete repression. 0.25  $\mu$ g

each of N-CoR and HDAC1 together could totally ablate transcriptional activity along with the low amount of prohibitin, suggesting that both these proteins are involved in prohibitin-mediated repression of E2F1. Even 2  $\mu$ g each of N-CoR or HDAC1 did not inhibit E2F1 in the absence of prohibitin (data not shown), suggesting that these molecules have to be recruited to the promoter by other proteins to effect repression.

Similar experiments were conducted using minimal amount of Rb instead of prohibitin. While increasing amounts of N-CoR, up to 1  $\mu$ g, could not synergize with Rb to repress E2F1, the lowest amount of HDAC1 tested (0.25  $\mu$ g) could partially repress E2F1 and 0.5  $\mu$ g could eliminate E2F activity completely. Co-transfection of low amounts of N-CoR did not enhance the ability of HDAC1 to synergize with Rb, indicating that N-CoR hardly contributes to the process. These results show that the functional effect of N-CoR and HDAC1 closely parallels their ability to associate with Rb and prohibitin physically: HDAC1 which binds to Rb can synergize with it, but N-CoR is unable to bind or synergize. But both the co-repressors can associate with prohibitin and synergize with it functionally.

#### Transcriptional repression by prohibitin coincides with histone deacetylation

Transcriptional activation normally follows acetylation of histones, mainly histones H3 and H4, while transcriptional repression correlates with histone deacetylation. Since prohibitin recruits HDAC1, it was next checked whether repression by prohibitin correlated with deacetylation of histones. Chromatin immunoprecipitation assays (CHIP assays) using antibodies to acetylated histone H3 (AcH3) were used for this purpose (Alberts *et al.*, 1998; Dedon *et al.*, 1991; Luo *et al.*, 1998). The first set of experiments was conducted on transiently transfected promoters. When E2CAT was transfected along with E2F1, CHIP assay could detect the association of the promoter with acetylated H3, correlating transcription with histone acetylation (Figure 4a). Upon co-transfection of prohibitin to repress E2F1, there was no detectable

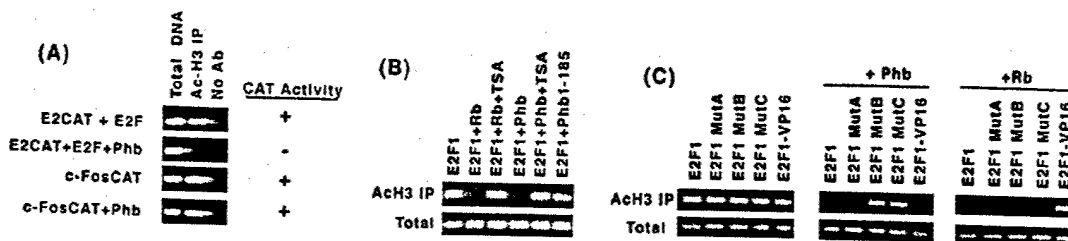


Figure 4 (a) CHIP assays on T47D cells transiently transfected with the indicated vectors. The PCR product detects the 5' end of the CAT gene. Total indicates a PCR on the same amount of DNA used for IP; CAT activity observed in the same lysates is indicated on the right. (b) CHIP assay on T47D cells as in (a), where the cells were treated with TSA in the indicated lanes after co-transfecting Rb and Phb. (c) The indicated mutants of E2F1 were co-transfected with Rb or prohibitin. In all the cases, absence of a band in the CHIP assay correlated with transcriptional repression

amount of DNA present in the AcH3 immunoprecipitate, suggesting that the histones have been deacetylated. A CHIP assay was performed on a c-Fos CAT reporter as control; here, co-transfection of prohibitin did not repress c-Fos promoter, and there was still a considerable amount of DNA detectable in the AcH3 immunoprecipitate. This suggests that the recruitment of HDAC1 by prohibitin is a specific event that occurs only on promoters that are regulated by prohibitin.

To confirm whether the absence of DNA in the AcH3 immunoprecipitate was indeed due to deacetylation, E2CAT and E2F1 were co-transfected along with Rb or prohibitin; CHIP assays show the ablation of DNA in the IP (Figure 4b). When the transfected cells were treated with 200 nM TSA, the transcriptional repression is relieved correlating with the appearance of DNA in the immunoprecipitate. Further, a mutant prohibitin which could not repress E2F activity did not bring about histone deacetylation. These results suggest that the absence of DNA in the AcH3 immunoprecipitate is due to the deacetylation of histones.

CHIP assays were designed to check whether prohibitin could bring about histone deacetylation when E2Fs lacking the marked box region was used. As shown in Figure 4c, DNA is associated with AcH3 when full length E2F1, or mutants lacking different regions of the marked box MutA (-283-304), MutB (-304-326) and MutC (-326-357) or E2F1-VP16 fusion is transfected. Upon co-transfection of prohibitin, DNA can be detected only when E2F1 304-326 (MutB) and E2F1 326-357 (MutC) are used. Since these E2F1 constructs are not repressed by prohibitin, there appears to be a direct correlation between the ability of prohibitin to repress transcription and to induce histone deacetylation. When Rb is used to repress the different E2F1 constructs, DNA can be detected only in the immunoprecipitate where E2F1-VP16 is used, which is not repressed by Rb. This shows that both Rb and prohibitin bring about histone deacetylation, despite targeting different regions of E2F1.

Since stimulating IgM receptors in Ramos cells can reverse prohibitin-mediated repression of E2F1, attempts were made to examine whether this alters the acetylation status of histones. Ramos cells were transfected with E2CAT and E2F1, and DNA could be detected in association with acetylated histones. As in T47D cells, co-transfection of prohibitin repressed E2F activity, eliminating the DNA associated with acetylated histones (Figure 5a). Stimulation of the transfected cells with an anti-IgM antibody led to a release of the repression along with the acetylation of histones as detected by the DNA present in the IP. Similarly, co-transfection of Rb also led to histone deacetylation, but treatment of the cells with an anti-IgM antibody did not release the repression, and there was no increase in histone acetylation. This result shows that the recruitment of histone deacetylases by prohibitin is dependent on the functional status of prohibitin, and signals that can inactivate prohibitin also abrogate prohibitin-mediated histone deacetylation.

Attempts were made to evaluate whether the changes in histone acetylation were true for endogenous promoters as well. This was examined by performing CHIP assays on control Ramos cells or those stably over-expressing prohibitin. Two promoters, Cdc25A and Rb, which are regulated by E2F binding sites, were tested. Both the promoters were found to be associated with acetylated histones in the control Ramos cells but not in the Ramos cells over-expressing prohibitin (Figure 5b). But upon IgM stimulation, there was DNA associated with AcH3, showing that histone deacetylases are no longer present on either promoter. No changes were observed on the endogenous c-Fos promoter, which is not regulated by prohibitin. Northern blot analysis show that the changes in the acetylation status of the promoters correlate with the expression of the message: both Cdc25A and Rb are expressed at very low levels in prohibitin over-expressing cells, but IgM treatment leads to transcription of both the genes (Figure 5c). There was no significant change in the expression levels of a control GAPDH gene. The expression of these promoters

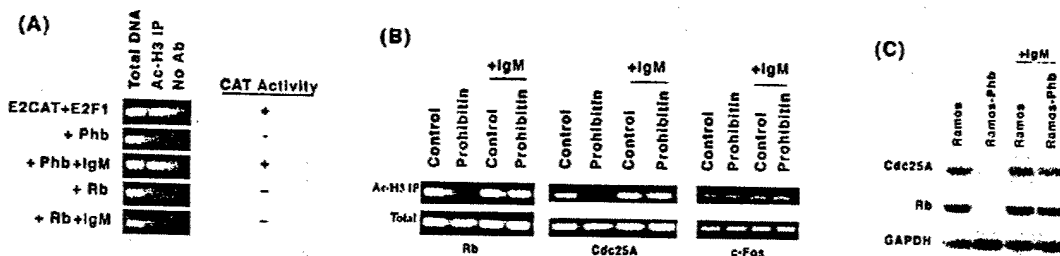


Figure 5 (a) CHIP assays were conducted on Ramos cells transiently transfected with the indicated vectors. Treatment of the transfected cells with an anti-IgM antibody reversed Phb-mediated repression and led to a band in the CHIP assay. (b) Control Ramos cells or those stably over-expressing prohibitin were treated with an anti-IgM antibody. CHIP assays were conducted on the indicated promoters using genomic DNA as the template. It can be seen that IgM stimulation reverses the repression of Rb and Cdc25A promoters, but has no effect on the c-Fos promoter. (c) A Northern blot analysis showing the levels of Rb and Cdc25A RNA in the same cells

parallels the transcriptional activation observed in transient transfection experiments and suggest that recruitment of histone deacetylases to promoters is one mechanism by which prohibitin represses endogenous promoters as well.

## Discussion

Regulation of transcription factor activity is effected at multiple levels – at the level of DNA binding, factor modification, interaction with other regulatory proteins, the recruitment of general transcription factors and the utilization of transcriptional co-activators and repressors. It has also become clear that the DNA template, especially the status of the nucleosomes and chromatin the promoter region, also contribute to the regulation (Collingwood *et al.*, 1999; Howe *et al.*, 1999; Luo and Dean, 1999; Xu and Rosenfeld, 1999). Regulation of E2F activity by Rb and its family members involve all these processes at some level (Brehm and Kouzarides, 1999; Harbour and Dean, 2000b). The results presented here show that the regulatory protein prohibitin represses E2F by utilizing histone deacetylases, but differ from Rb in that additional co-repressors are involved. In addition, our results show that a portion of total prohibitin is localized in the nucleus, and its membrane tethering domain is not necessary for its growth suppressive or transcription regulatory effects.

Though prohibitin was originally cloned based on its ability to suppress cell proliferation, its mechanism of action was not clear. The status of prohibitin gene/gene products in cancer is not clear either; studies have shown it to be a potential tumor suppressor, which is supported by the fact that it is a strong suppressor of cell proliferation. In contrast, it has been reported that prohibitin protein levels are elevated in human melanomas as well as testicular seminoma sections (Coates *et al.*, 2001). The same study suggests that prohibitin is localized to the mitochondria and responds to mitochondrial stress; but the immunofluorescence data presented in that study shows a significant amount of prohibitin in the nucleus, where its staining overlaps markedly with propidium iodide staining. The results presented in this paper shows that prohibitin is distributed in the nuclear and cytoplasmic compartments and the amino-terminal domain that supposedly localizes prohibitin to the mitochondria is not needed for its growth suppressive effects. Our previous studies had shown that the binding of prohibitin to Rb and E2F1 is necessary for its transcriptional repression and growth control. Prohibitin was found complexed with either Rb or E2F1 in different cell types including Ramos cells without overexpression of any component, as detected by immunoprecipitation–Western blot experiments (Wang *et al.*, 1999a,b). Because the Rb binding domain and the E2F binding domain on prohibitin are distinct (amino acids 74–116 versus 184–214, respectively), the three proteins could potentially associate together at the

same time. Our results on a transcriptional regulatory role for prohibitin are further supported by the studies showing that a highly related protein, Phb2/BAP37/REA mediates the repression of estrogen receptors (Delage-Mourroux *et al.*, 2000; Montano *et al.*, 1999). Collectively, these observations suggest that while prohibitin might have other functions in the cell (Nijtmans *et al.*, 2000, 2002; Steglich *et al.*, 1999), its mitochondrial functions, if any, are distinct from its ability to bring about transcriptional repression or growth suppression.

We had found that prohibitin could repress the transcriptional activity of E2Fs1–5, through the highly conserved marked box region. The studies presented here show that a sub-domain within this region is sufficient for prohibitin-mediated repression. Interestingly, this region of E2F1 can make unrelated factors like GAL4 and VP16 sensitive to prohibitin mediated repression. It seems possible that prohibitin interacts with these fusion proteins to recruit additional co-repressors. Our results also show that prohibitin is capable of repressing transcription when recruited to a promoter by different means: either through binding to E2F, or when fused to a GAL4 DNA binding domain, as has been shown for Rb.

Recruitment of prohibitin to promoters leads to HDAC1-dependent transcriptional repression suggesting that the mode of repression is similar to one used by Rb. Involvement of N-CoR in the repression process, though, introduces an intriguing divergence in the precise molecular mechanism. Involvement of N-CoR in prohibitin-mediated, but not Rb-mediated repression, provides one additional node where signals can preferentially target prohibitin-regulated promoters. Sin3A, though found in association with prohibitin in the co-immunoprecipitation assay, did not functionally synergize with prohibitin or Rb. We had previously shown that certain signals like E1A and cyclin-dependent kinases cannot relieve prohibitin-mediated repression of E2F. It is plausible that this is due to the involvement of a larger, high affinity repressor complex involving prohibitin. Various other co-repressors like DNMT1, CtIP/CtBP, HP1 and Ring1 (Dahiya *et al.*, 2001; Meloni *et al.*, 1999; Nielsen *et al.*, 2001; Robertson *et al.*, 2000), as well as chromatin remodeling proteins like Brg/Brm are also involved in Rb-mediated repression of various cellular promoters (Harbour and Dean, 2000a). It remains to be seen whether these co-repressors are involved in prohibitin-mediated transcriptional repression as well.

Stimulation of Ramos cells with an anti-IgM antibody was shown to preferentially rescue prohibitin-mediated repression of E2F1 activity (Wang *et al.*, 1999b). We had observed that this coincides with a dissociation of prohibitin, but not Rb, from E2F1. Here we show that deacetylation of histones mediated by prohibitin is negated by IgM stimulation. This is most likely due to the dissociation of prohibitin and associated HDAC1 from E2F. Strikingly, IgM does not affect Rb-mediated repression of E2F1 and there is

no alteration in the histone acetylation status in a transient transfection experiment. It is notable that IgM stimulation of control Ramos cells and those stably over-expressing prohibitin have different levels of histone acetylation on both the E2F-regulated promoters we examined. This correlated well with the expression of the two promoters as well. It appears that prohibitin represses the transcription of a variety of cellular genes and molecules that can modify histones facilitate this process.

It may be concluded that prohibitin is a potent inhibitor of E2F activity, and this inhibition can respond to specific extra-cellular signals. The actual mechanism of inhibition involves the action of histone deacetylases and co-repressors like N-CoR. The ability of prohibitin to target all the five transcriptionally active E2Fs might enable the cells to target a different set of genes than those regulated by Rb, since Rb can modulate the activity of only E2Fs1-3. In addition, the ability of prohibitin to respond to molecules that cannot affect Rb facilitates the cells to respond to a wider array of signals to elicit the appropriate biological response. Prohibitin thus appears to constitute a different tier of regulation of E2F activity and cell proliferation.

#### Materials and methods

##### Cell lines

The human breast carcinoma cell line T47D and the human primary fibroblast HSF-8 cell line were maintained in DMEM media supplemented with 10% FBS. The human B cell lymphoma Ramos cell line was maintained in RPMI media supplemented with 10% FBS.

##### Chromosome immunoprecipitation (CHIP) assays

CHIP assays were conducted using published protocols (Luo et al., 1998). Briefly, control or transfected cells were treated with formaldehyde (final concentration 1%) for 10 min at room temperature. Cells were washed with PBS and resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris HCl, pH 8.1, 1 mM PMSF, 1 mM Pepstatin A, 1 mM aprotinin) and sonicated. The samples were centrifuged at 14K, 4°C, for 5 min and the supernatant was divided into three: one-third was used to perform a control PCR for the total amount of plasmid transfected (or total amount of genomic DNA). Equal amount of the remaining DNA was immunoprecipitated with a control antibody or antibodies to AcH3 (UBI) in a buffer containing 0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris HCl pH 8.1 and 150 mM NaCl. The antibody bound complexes were recovered on protein A beads and protein/DNA was eluted in 300 µl of elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>). Crosslinking was reversed by heating at 65°C for 4 h. The DNA is resuspended in 200 µl of water, treated with 40 µg of proteinase K at 37°C for 30 min, followed by phenol/chloroform extraction and ethanol precipitation. PCR was done using 20-100 ng (500-1000 ng for genomic DNA) of DNA as template.

The following PCR primers were used for CAT gene, Rb promoter, Cdc25A promoter and cFos promoter. CAT: size of PCR product: 205 bp 5' primer: ACCACCGTGTATGATA-

TATCC; 3' primer: TTGCCATACGGAGTTCCG. Cdc25A promoter: size of PCR product: 209 bp 5' primer: TCTGCTGGGAGTTTTCATTGACCTC; 3' primer: TTGGCGCCAAACGGAATCCACCAATC. Rb promoter: size of PCR product: 194 bp 5' primer: TTCTATCTTCTAAGTGACTGG; 3' primer: GGTCTGATAGGGAAGACTCTC. c-Fos promoter: size of PCR product: 209 bp 5' primer: TGTTGGCTGCAGCCCGCAGCAGTTC; 3' primer: GGCGGTGTCCTAATCTCGTGAGCAT.

##### Transient and stable transfections

T47D cells were transfected by calcium phosphate precipitation by using standard protocols (Wang et al., 1999b). Ramos cells were transfected with pCDNA3-prohibitin by electroporation as described before. Individual clones stably expressing prohibitin were obtained by constant neomycin selection at 40 µg/ml, the resulting clones were confirmed by Western blotting. Ramos cells were treated with goat anti-human IgM antibody (Southern Biotechnology Associates, Inc.) at 1 µg/ml for 4 h before harvesting. Constructs pDCE2F1, pE2CAT, pSVRb, pCDNA3-prohibitin have been described before (Wang et al., 1999a; Zhang and Chellappan, 1995). PCMX.N-CoR was a gift from Dr Scott W Hiebert, pBJ5-HDAC1-F is a kind gift from Dr Robin Luo and Dr Douglas Dean. pMLPGAL4-CAT, pCDNA3GAL4.Rb, pSVECGCAT were kind gifts from Dr Joseph Nevins. Deletion mutants of E2F1 were generated by PCR fused to GAL4DBD, VP16AD or GAL4VP16 by overlap extension PCR and cloned in pCR3.1 vector. GAL4-prohibitin was generated by a similar overlap-extension protocol. CAT assays were done using standard protocols (Sambrook et al., 1989).

Stable transfections were performed on 35 mm dishes using approximately 10 000 cells and subjected to selection in the appropriate antibiotic for 14 days. The total amount of DNA transfected was equalized using salmon sperm DNA in every sample. Cells were fixed and stained with crystal violet and colonies having more than 20 cells were counted.

##### Immunofluorescence, immunoprecipitation and Western blot analysis

Double immunofluorescence experiments on human primary fibroblast HSF-8 cells were carried out using previously described protocols (Wang et al., 1998) and cells visualized by confocal microscopy using a Perkin Elmer Spinning Disc Confocal Imaging system mounted on a Nikon TE200 microscope. Anti-prohibitin mouse monoclonal antibodies were obtained from Neomarkers Inc., and anti-Rb rabbit polyclonal antibodies from Santa Cruz Biotechnologies. Whole cell extracts were immunoprecipitated with N-CoR, HDAC1 or Sin3A antibody (from Santa Cruz Biotechnology) using published protocols (Wang et al., 1998, 1999b). Western blot analysis using Rb monoclonal antibody (Calbiochem) and prohibitin antibody (from Neo Markers) was done using standard protocols and visualized by the ECL system (Amersham).

##### Northern blot analysis

Total RNA was prepared from Ramos cells followed by Northern blot analysis by using standard protocols (Fusaro et al., 2002). Rb, Cdc25A and GAPDH probes were synthesized by using Prime-a-Gene Labeling System (Promega), and used to probe 10 µg of RNA. The bands were visualized by autoradiography.

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